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Isolation and Serial Propagation of Human Group C Rotaviruses in a Cell Line (CaCo-2)

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Rotaviruses were detected via electron microscopy in fecal specimens collected from school children during an outbreak of diarrhea and from a sporadic case in 1993 in Japan. All of the viruses were found to belong to human group C rotavirus by reverse passive hemagglutination assay (RPHA). These viruses replicated well in a human colon carcinoma (CaCo-2) cell line cultured in the presence of trypsin (4 μg/ml). This report demonstrates that human group C rotaviruses can be propagated efficiently in a cell line cultured in the presence of trypsin. The infected cells did not show any apparent cytopathic changes. However, virus was detected in the cell cytoplasm by immunofluorescence (IF) staining and in the culture supernatant by RPHA. On the basis of immune electron microscopy (IEM), virus particles collected from infected CaCo-2 cell cultures were confirmed to aggregate specifically with anti-human group C rotavirus antibody. The electrophoretic patterns of RNA segments extracted from viral particles found in the fecal specimens or infected cells were identical to those of human group C rotavirus. These results indicated that human group C rotaviruses were the causal agent of the diarrhea outbreak.

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KEY WORDS: gastroenteritis, immune electron micrography, reverse passive hemagglutination, trypsin, viral diarrhea

INTRODUCTION

Rotaviruses are a major causal agent of gastroenteritis in infants and young children. In addition, viruses called pararotaviruses or atypical rotaviruses, which are morphologically identical, have been found in humans and other animals, but they are antigenically distinct from typical rotaviruses. At present, typical rotaviruses are classified as group A and atypical rotaviruses as groups B–G [Saif, 1990].

Group C rotaviruses were first identified as a causal agent of gastroenteritis in swine in 1980 [Saif et al., 1980] and in humans in 1982 [Rodger et al., 1982]. Human group C rotavirus infections, however, have been reported only occasionally in many countries [Dimitrov et al., 1983; Espejio et al., 1983], including Japan, where only a few outbreaks have been reported [Matsumoto et al., 1989; Ohseto, 1990].

Group A rotaviruses as well as porcine [Saif et al., 1988] and bovine [Tsunemitsu et al., 1991] group C rotaviruses can be propagated serially in cell cultures. However, attempts to propagate other group C rotaviruses or other atypical rotaviruses have failed. In this paper, we demonstrate that human group C rotaviruses can be propagated efficiently and passaged serially in a human colon carcinoma cell line cultured in the presence of trypsin.

MATERIALS AND METHODS

Fecal Specimens

Fecal specimens were obtained from seven patients. Six of the specimens (291–296) were obtained from children with diarrhea during an outbreak at a primary school. One of the specimens (360) was obtained from a 4-year-old boy with a sporadic case of diarrhea. This specimen was collected at the same time in another city distinct from the primary school during April of 1993, in Chiba Prefecture, Japan.

Viruses and Cell Cultures

The rotavirus Wa strain was propagated in MA104 cells and used as a reference strain for group A rotavirus. The continuous human colon carcinoma cell line CaCo-2 [Willcocks et al., 1990] was cultured in roller tubes using a drum culture system. The drum was set to rotate once per minute. The cells were cultured with Eagle's minimum essential medium (MEM) containing 5% fetal calf serum, 0.15% sodium bicarbonate, 1 μg/ml

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fungizone, and antibiotics (400 IU/ml penicillin, 200 μg/ml streptomycin, 200 μg/ml kanamycin).

Virus Propagation

The fecal specimens were homogenized to a 10% suspension in phosphate-buffered saline (PBS) and centrifuged at 1,700*g* for 20 min. The supernatant was collected and used as virus suspension after the addition of antibiotics and fungizone to the same concentration as in the MEM. One hundred microliters of the virus suspension was used to inoculate CaCo-2 monolayers, which were washed three times in PBS. After adsorption for 60 min at 37°C, the monolayers were washed twice in PBS, and 1 ml of serum-free MEM containing a designated amount of trypsin (Sigma; bovine pancreas Type 1) was added to the cultures. The cultures were incubated in the roller drum for 5 days at 37°C and subjected to one freeze-and-thaw treatment in order to make a virus suspension. Subsequent virus passages were carried out in an identical manner using 100 μl of virus inoculum obtained from infected cell cultures.

Indirect Immunofluorescence (IF) Staining

Infected cell suspensions (4 days postinfection) were smeared on a glass slide, then air-dried and fixed in 100% acetone. The fixed slides were incubated with guinea pig antiserum against human group C rotavirus for 30 min at 37°C. They were then washed three times in PBS, air dried, and stained with fluorescein isothiocyanate (FITC)-conjugated rabbit antibody to guinea pig IgG for 30 min at 37°C. The degree of immunofluorescence was scored as +1, +2, or +3 if approximately 10%, approximately 25%, or more than 50% cells were stained, respectively. The guinea pig antiserum (86-542) to human group C rotavirus was kindly supplied by M. Ohseto [Ohseto, 1990], Public Health Laboratory of Ehime Prefecture. The antiserum was obtained by immunizing guinea pigs with group C rotaviruses isolated from an infant in Ehime, Japan, in 1986. The virus antigen was confirmed to be reacted only with porcine group C rotaviruses [Nakata et al., 1986].

Electron Microscopy (EM) and Immune Electron Microscopy (IEM)

Virus samples for EM were prepared by a slight modification of the method of Bishop et al. [1974]. Briefly, 10 ml of a 10% suspension of fecal specimens in PBS was mixed with an equal volume of trichlorotrifluoroethane and centrifuged at 1,700*g* for 20 min. The aqueous layer was collected, layered onto a 30% (weight/volume in water) sucrose cushion, and centrifuged at 110,000*g* for 2.5 hr at 4°C. The pelleted virus particles were resuspended in 100 μl distilled water and used for EM and IEM. The virus samples were placed on grids and negatively stained with 2% phosphotungstic acid (PTA) and examined using a JEM1010 electron microscope (JEOL, Japan). IEM was carried out by the method of Kapikian et al. [1972]. Briefly, 25 μl of the purified virus suspension was mixed with 25 μl of the antiserum 86-542 (diluted 1:100) against human rotaviruses, incubated for 1 hr at 37°C, and stained with 2% PTA prior to IEM.

TABLE I. Identification of Viruses From Fecal Specimens by ELISA, RPHA, and IF*

Specimen No.	ELISA[a]	RPHA titer[b]	IF staining
291	–	1,024	+1
292	–	1,024	+1
293	–	2,048	+2
294	–	2,048	+1
295	–	256	+1
296	–	2,048	+2
360	–	1,024	+1

*Fecal specimens were twofold serially diluted with PBS, and RPHA titers were determined as the reciprocal number of the final positive dilution. Each specimen was inoculated into CaCo-2 cell cultures containing 4 μg/ml trypsin, and cultures were fixed with acetone 4 days after inoculation. The fixed cells were stained with IF antibody (86-542). The intensity of IF staining was expressed as +1, +2, or +3 as described in Materials and Methods.
[a]Detection for group A rotavirus.
[b]Detection for group C rotavirus.

Polyacrylamide Gel Electrophoresis

Genomic RNAs were released from viral particles purified from fecal specimens or infected cell cultures by phenol-chloroform extraction. The RNAs were separated by electrophoresis using a 5–10% polyacrylamide gel at 20 mA for 3 hr, then stained using a Silver Staining Kit (Bio-Rad Laboratories).

Enzyme-Linked Immunosorbent Assay (ELISA) and Reverse Passive Hemagglutination Assay (RPHA)

ELISA was carried out using a kit (Rotaclone; Cambridge Biotech Co.) to detect group A rotaviruses. RPHA was carried out according to the method of Kuzuya et al. [1993] using a detection kit for group C rotaviruses. This kit was kindly supplied by R. Fujii, Okayama Prefectural Institute for Environmental Science and Public Health.

RESULTS

An outbreak of acute gastroenteritis was seen among children in the second through the sixth grades in a primary school in Chiba, Japan, in 1993. The frequency of acute gastroenteritis was reported to be 25.2% (187 of 743 children) in the school. The clinical symptoms were abdominal pain (60.2%), nausea (55.9%), vomiting (36.0%), diarrhea (47.1%), and a fever of more than 37°C (37.5%).

Rotavirus-like particles were detected in 30 of 36 fecal specimens collected from the children by EM. All of these particles were confirmed to be human group C rotaviruses, and not group A rotaviruses, by ELISA and RPHA using specific anti-human rotavirus antibodies. Seven of the identified viruses are presented in Table I. These results revealed that the outbreak of viral diarrhea in the school children was the result of group C rotavirus infection.

TABLE II. Trypsin Effect on Human Group C Rotavirus Propagation in CaCo-2 Cells*

Virus	RPHA titer (μg/ml)[b]			IF intensity (μg/ml)		
	1	2	4	1	2	4
293C-2[a]	8	16	64	+1	+2	+2
294C-2	<8	<8	<8	−[c]	+1	+1
296C-2	<8	16	16	−	+2	+2

*Viruses were inoculated into CaCo-2 cell cultures grown in culture medium containing 1, 2, or 4 μg/ml trypsin. The infected cells and culture supernatant were collected for IF and RPHA, respectively, at 4 days after inoculation.
[a]Viruses were passaged twice in CaCo-2 cells before inoculation.
[b]Trypsin concentration in culture medium.
[c]Negative result.

To characterize these viruses further, an attempt was made to propagate them in tissue cultures grown in the presence of trypsin. Partially purified virions from seven (see Table I) of the fecal specimens were inoculated into CaCo-2 cells. The supernatants from all of the virus-infected cultures were positive for virus replication based on IF using an anti-human group C rotavirus antibody. To determine the appropriate concentration of trypsin for optimal virus propagation, CaCo-2 cell cultures grown in the presence of 1, 2, or 4 μg/ml trypsin were inoculated with viruses. As is shown in Table II, the highest RPHA titer and IF intensity were obtained when 4 μg/ml of trypsin was added to the culture. When more than 4 μg/ml of trypsin was added to the cultures, the cells easily detached from the tubes, resulting in very low virus yields (data not shown). Therefore, the culture medium was supplemented with 4 μg/ml trypsin, the most effective dose for virus propagation in subsequent experiments.

To examine virus replication, CaCo-2 cells were inoculated with 32, 3.2, or 0.3 units of virus, based on RPHA, and the culture supernatant was collected (Fig. 1). In cultures infected with 32 RPHA-based units, the viruses replicated exponentially from day 2 to day 4; however, in cultures in which the inoculum was reduced to 1/10, i.e., 3.2 units, a 1 day lag in replication was found. At a multiplicity of infection of 32 RPHA units, the infected cells did not display any detectable cytopathic changes compared with uninfected cells.

At 4 days after inoculation, cells infected with 32 RPHA units of virus were harvested and stained by IF. As is shown in Figure 2, most cells were stained positively although the intensity of staining varied. As is shown in Figure 3, virions of virus 293C-5 (the 293C-5 designation indicates that the virus 293 isolated from fecal specimen was passaged five times in CaCo-2 cells), which were collected from infected CaCo-2 cells at 4 days after inoculation and purified by ultracentrifugation, were aggregated by anti-human group C rotavirus serum in a manner similar to that seen for viruses purified from the original fecal specimen. Also, few empty virions were observed, suggesting that the viruses replicated efficiently in CaCo-2 cells with the presence of trypsin.

Viral genomic RNA segments were analyzed by polyacrylamide gel electrophoreses. The electrophoretic migration patterns of viruses 293 and 293C-6 were typical of human group C rotaviruses [Saif, 1990] (Fig. 4).

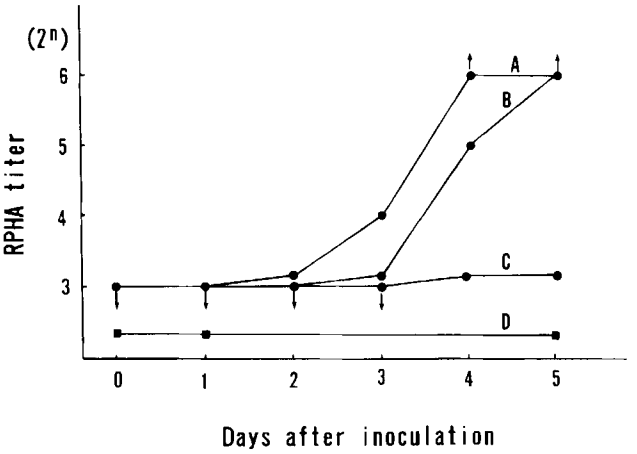

Fig. 1. RPHA titers of CaCo-2 cell culture fluid infected with virus 293C-6 (i.e., virus 293 passaged 6 times in CaCo-2 cells). CaCo-2 cells were inoculated with undiluted (32 RPHA units; A), 1/10 diluted (3.2 RPHA units; B), or 1/100 diluted (0.3 RPHA units; C) virus 293-C6 on day 0. The bottom curve (D) indicates uninfected control culture fluid. A supernatant sample was collected from each culture every day, and the RPHA titer of each sample was determined. The arrows indicate a titer of more than 2^6 or less than 2^3.

DISCUSSION

We have demonstrated that human group C rotaviruses were a causative agent of an outbreak of diarrhea in school children in Japan. In the 1970s, most outbreaks of viral diarrhea in schools were reported to be caused by group A rotaviruses [Hara et al., 1976]. Recently, several viral agents have been reported to be the causative agent of diarrhea outbreaks, including group B rotaviruses found to be responsible for several large outbreaks in China [Hung et al., 1983, 1984; Chen et al., 1985]. In Japan, small round viruses are identified most frequently as a causative agent of diarrhea outbreaks [Hayashi et al., 1989; Sekine et al., 1989]. On the other hand, group C rotaviruses are known as causative agents of gastroenteritis in bovine species [Saif et al., 1980], but only a few sporadic cases have been attributed to this virus in humans [Dimitrov et al., 1983; Espejio et al., 1984; Ohseto, 1990]. Our data, how-

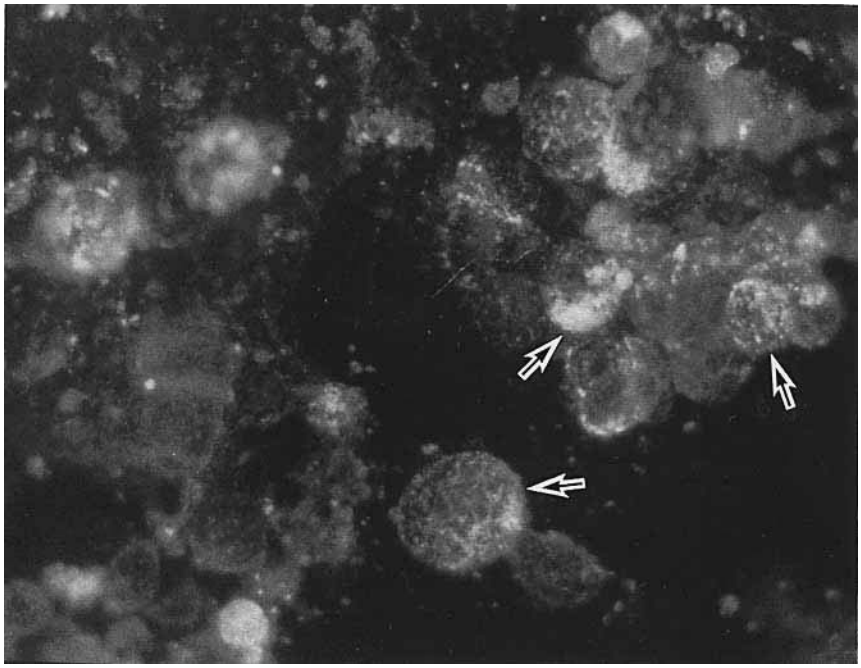

Fig. 2. IF staining of CaCo-2 cells infected with virus 293C-5. The cells were stained at 4 days after infection. Arrows indicate specific fluorescence.

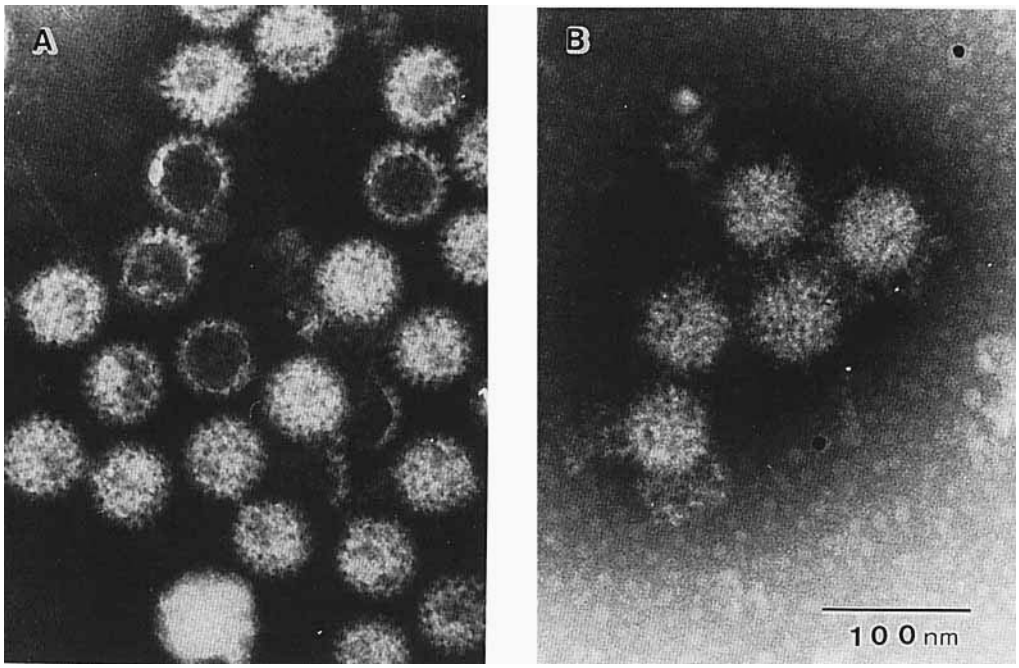

Fig. 3. Virions collected from a fecal specimen and cell culture fluid. **A:** EM of partially purified virions obtained from fecal specimen 293. **B:** IEM of virus 293C-5. The collected viruses were purified, concentrated, and incubated with anti-human group C rotavirus antibody (86-542).

ever, suggest that group C rotaviruses may play an important role in outbreaks of viral diarrhea.

In previous studies, rotavirus replication in tissue culture has been limited mostly to group A viruses. Only two reports of the replication of group C rotaviruses, one isolated from swine and the other from a bovine source, have been published [Saif et al., 1988; Tsunemitsu et al., 1991]. We propagated group C rota-

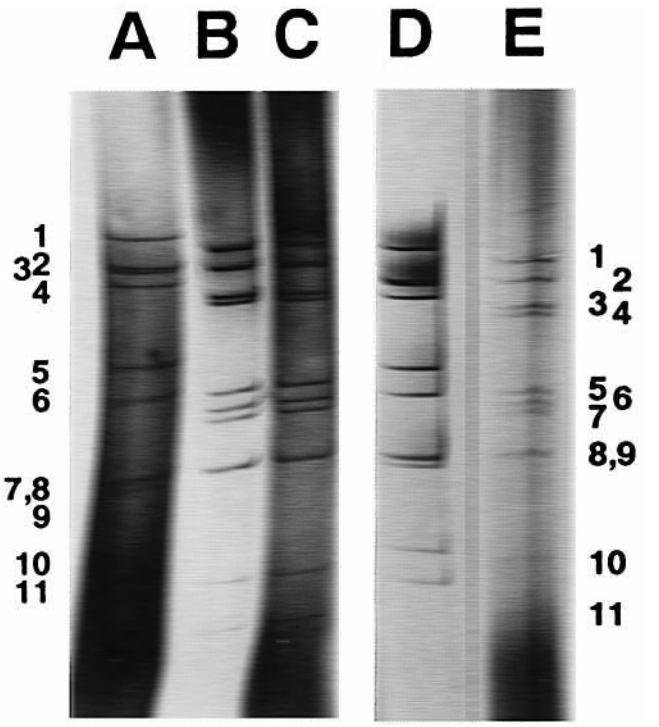

Fig. 4. Comparison of the electrophoretic migration patterns of rotavirus double-stranded RNA segments. The segments were electrophoresed in a 5–10% polyacrylamide gel, and the gel was stained with silver nitrate. **Lanes A** and **D:** Human group A rotavirus (Wa strain). **Lanes B** and **C:** Virus 293 and virus 296 (directly from fecal specimen). **Lane E:** Virus 293C-6 (the fecal specimen 293 passaged 6 times in CaCo-2 cells). The numbers 1–11 indicate the different RNA segments of the rotavirus genome.

viruses originating from humans using CaCo-2 cell cultures, which have been shown to support human astroviruses replication [Willcocks et al., 1990]. The highest rotavirus yields were obtained when the cells were cultured in a rotating system and in medium containing 4 μg/ml trypsin. This cell system produced highly infectious virions, even though trypsin treatment may cleave the major outer capsid polypeptides of the virions, as was demonstrated with group A rotaviruses by Estes et al. [1981]. CaCo-2 cells are useful, therefore, for the study of atypical rotaviruses, including group C viruses, which are important epidemiologically.

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